Chem. Res. Toxicol. **1998,** *11,* 136−142

Stable Acetaldehyde−Protein Adducts as Biomarkers of Alcohol Exposure

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Received September 15, 1997

The consumption of alcoholic beverages has been associated with increased risks of a number of chronic disorders including cancers. It is still not clear whether ethyl alcohol or other components such as metabolites are directly involved in the carcinogenic process or whether the effects are due to the modulation of metabolism of other carcinogens. At present, there is no good biomarker of alcohol intake, particularly at low or moderate levels of consumption. A number of studies have shown the ability of the major metabolite acetaldehyde to react with proteins in vitro to give stable and unstable adducts. The interaction of acetaldehyde with model peptides, which correspond to N-terminal globin sequences, was studied. The major stable adduct was identified by mass spectrometry and NMR as a diastereoisomeric mixture of imidazolidinones. This is believed to be formed by reaction and cyclization of the initial Schiff base adduct with the N-terminal valine. Incubation of human globin with acetaldehyde (0−2 mM) yielded products which were identified as the N-terminal adducts by electrospray ionization mass spectrometry (ESI-MS) of proteolytic digests. The specificity and sensitivity of the analysis was improved by the use of on-line HPLC−ESI-MS. Tryptic digests of the modified globin which contained both the N-terminal acetaldehyde adducts of α-globin (heptapeptide) and β-globin (octapeptide) were resolved. These results suggest that analysis of stable imidazolidinone adducts is a promising approach to estimation of alcohol exposure.

Introduction

The health risks linked to chronic alcohol consumption include damage to liver (*1, 2*) and brain (*3*), stomach disorders, and depression, as well as cancers of the upper aerodigestive tract, stomach, and liver (*4, 5*). The biochemical processes involved in the mechanism of damage have not yet been established, although the interaction of ethanol's metabolites and other chemicals have been cited as possible causes. Early detection of alcohol misuse before the manifestation of some of the damage mentioned earlier is at present not easy to determine. Hence there has been a considerable interest in the development of markers of alcohol exposure, particularly at moderate to excess levels of consumption.

When an alcoholic beverage is consumed, the alcohol is enzymatically converted to acetaldehyde and then to acetic acid. When ingested, alcohol is quickly absorbed in the gastrointestinal tract and 90% of this is metabolized in the liver. Production of acetaldehyde, the first metabolite in the hepatocytes, is catalyzed by alcohol dehydrogenase (ADH)[1] or by the microsomal ethanol-oxidizing system (MEOS) which is dependent on CYP2E1 (*6*). Further oxidation by the enzyme aldehyde dehydrogenase (ALDH) produces a harmless product, acetate. Acetaldehyde, however, is highly reactive due to the electrophilic nature of the carbonyl carbon and has been shown to form adducts with various proteins (*7−9*), DNA (*10*), and nucleosides (*11, 12*). Accumulation of acetaldehyde due to excess consumption of alcohol will therefore lead to increased interaction of this chemical with biomolecules.

There have been a number of reports which show that acetaldehyde forms stable and unstable adducts with hemoglobin and other proteins (*13, 14*) which could be used as biomarkers of exposure. The main groups on proteins which have been identified as target sites for adduction with acetaldehyde are α-amino groups (*15*), ε-amino groups (*16*), and thiol groups (*17*). In the case of hemoglobin, several types of adducts have been identified that are due to modification by acetaldehyde. Irreversible products have been identified at the N-terminal end of hemoglobin as a result of stabilization of the Schiff base by cyclization to produce the imidazolidinone (*15, 18*). Scheme 1 shows the mechanism of the reaction with N-terminal peptides. Schiff bases are formed reversibly and can be stabilized by reduction. Under in vivo conditions it is believed that ascorbate can be the reducing agent (*19*). Excess NADH produced in the metabolism of ethanol and acetaldehyde has also been proposed as a potential reductant to stabilize these adducts (*20*).

This study has focused on the development of methods for detection of adduction of acetaldehyde with the N-terminal amino group of hemoglobin since initial

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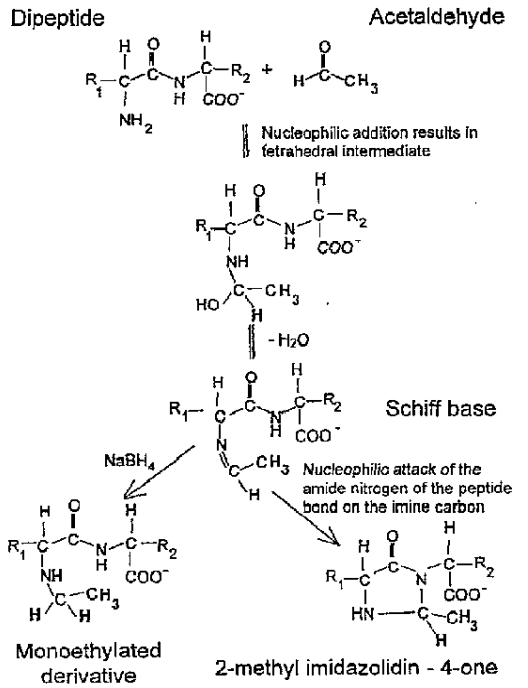
[1] Abbreviations: ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase; bis-Tris, 2,2-bis(hydroxymethyl)-2,2′,2″-nitrilotriethanol; DSS, sodium 2,2-dimethyl-2-silapentane-5-sulfonate; ELISA, enzyme-linked immunosorbent assay; ESI-MS, electrospray ionization mass spectrometry; HbA, normal hemoglobin; MEOS, microsomal ethanol-oxidizing system; MS/MS, tandem mass spectrometry; PEG, poly(ethylene glycol); SIR, selective ion recording; TFA, trifluoroacetic acid; TIC, total ion current; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone.

S0893-228x(97)00169-0 CCC: $15.00
Published on Web 01/23/1998

Scheme 1. Reaction of Peptide with Acetaldehyde Showing Formation of Intermediate Schiff Base Followed by Cyclization under Nonreducing Conditions To Give Imidazolidinone[a]



[a] R1 and R2 represent the side chains. Adapted from San George and Hoberman (*15*).

experiments with model peptides suggested that the adduct was stable and could be the basis for a quantitative biomarker assay for alcohol exposure.

Materials and Methods

HPLC solvents were purchased from Fisher Scientific, U.K., and all other chemicals from Sigma Chemical Co., Dorset, U.K., unless otherwise stated.

Peptides. The following peptides were synthesized by the Protein and Nucleic Acid Sequencing laboratory, CMHT, University of Leicester: (A) 21-mer polypeptide, Val-Leu-Ser-Pro-Ala-Asp-Lys-Thr-Asn-Trp-Gly-His-Glu-Tyr-Arg-Met-Phe-Cys-Gln-Ile-Gly; (B) heptapeptide, Val-Leu-Ser-Pro-Ala-Asp-Lys; (C) N-terminal α-peptide, Val-Leu-Ser-Pro-Ala-Asp-Lys-Thr-Asn-Val-Lys-Ala; and (D) N-terminal β-peptide, Val-His-Leu-Thr-Pro-Glu-Glu-Lys-Ser-Ala-Val-Thr. The following peptide was purchased from Sigma: (E) dipeptide, Val-Leu.

Globin Preparation. Fresh blood obtained in heparinized tubes was spun at 2000 rpm for 15 min. After removal of the plasma supernatant, the red blood cells were washed with saturated PBS, pH 7.3, and lysed by addition of an equal volume of water. The resultant lysate was dialyzed against distilled water overnight with three changes of the solution. Centrifugation at 25000*g* removed the cell debris. The globin was precipitated by addition of cold acidic acetone (1% HCl). The precipitate was washed four times with acetone–diethyl ether (1:1, v/v) followed by two more washes with diethyl ether. The globin was allowed to dry in air.

Reaction of Peptides and Globin with Acetaldehyde. To avoid loss of acetaldehyde during pipetting of the chemical, the samples, pipets, tips, syringes, and all other materials were placed in the cold room for at least 30 min to equilibrate to ambient temperature (4 °C). Peptides (1–2 mg/mL) were incubated with excess acetaldehyde in molar ratios of 1:50–100 in 20 mM 2,2-bis(hydroxymethyl)-2,2′,2″-nitrilotriethanol (bis-Tris) buffer at pH 6.5 in a water bath at 37 °C for up to 24 h.

Globin experiments were performed using samples prepared in different ways (Sigma; nondialyzed globin prepared in the laboratory from human blood and a dialyzed version of the latter). For the nondialyzed globin, 30 mg/mL samples were typically prepared in the 20 mM bis-Tris buffer (pH 8–9). Due to the trapped acid (HCl) from the preparation of the globin, the pH of the mixture had to be adjusted with dilute ammonia solution up to pH 6.5 or until just before precipitation. For Sigma and dialyzed globin, 2–3 mg/mL samples were dissolved in the buffer and required no further adjustment to pH. Aliquots of acetaldehyde from a freshly prepared 1 M stock solution (or dilutions thereof) were added to each tube to give final concentrations of 2–200 mM. Two control samples in the form of globin only and buffer plus acetaldehyde were also prepared. All samples were rapidly brought to 37 °C in a water bath. After the allotted incubation times (0–55 h), the samples were dried in either a centrifugal vacuum concentrator (Speed-Vac, Savant Instruments, Inc.) or a freeze-drier (Lyoprep-3000, International Equipment Co.) to remove the unreacted acetaldehyde.

Tryptic Digest of Peptides and Globin. The dried samples from the incubations were dissolved in 50 mM ammonium bicarbonate buffer (pH 8.5) at a concentration of 2–3 mg/mL. Freshly prepared trypsin (TPCK-treated) was added, at a substrate:enzyme ratio of 25:1 (w/w), and all samples were incubated at 37 °C. The digestion times varied from 0 to 24 h. The digestion was terminated by addition of an equal volume of 1% TFA solution. The samples were then immediately frozen in liquid nitrogen for freeze-drying.

HPLC Analysis of Peptides. The peptides were purified by reversed-phase HPLC on a Gilson instrument using a Spherisorb C18 column and a mobile phase of 0.1% TFA (solvent A) and 0.08% TFA in 80% acetonitrile (solvent B). A linear gradient from 5% to 80% B in 30 min at a flow rate of 1 mL/min was used. Detection was by a 1000S diode array UV detector (Applied Biosystems) at 215-nm absorbance. Samples were collected, dried, and redissolved in 0.08% TFA in 80% acetonitrile mixture for MS analysis.

HPLC Analysis of Globin. The HPLC separation was carried out on the same instrument as for the peptide analysis using an Aquapore butyl column (7 μm, 220 × 4.6 mm, Brownlee column; Applied Biosystem) using the same solvents. Separation of the α- and β-chains of globin was achieved using the gradient system from 45.0% to 75.0% solvent B in 30 min at a flow rate of 1.5 mL/min. Separation of the digests was achieved using the same column but a slightly different gradient of 5.0–80.0% solvent B in 30 min at a flow rate of 1.5 mL/min. Detection for both systems was at 215- and 280-nm absorbance.

NMR. Proton (^{1}H) NMR analysis was recorded on a Bruker ARX 250-MHz instrument. Samples were dissolved in D_2O with sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) as reference.

ESI-MS. Samples were analyzed on a Quattro-BQ triple quadrupole MS (Micromass, Manchester, U.K.) using a 1:1 mobile system of 0.1% TFA and 0.08% TFA in 80% acetonitrile, which was delivered at a flow rate of 50 μL/min using a Varian pump (Walton on Thames, U.K.). Samples were introduced into the MS via a Rheodyne injector with a 10-μL loop or by infusion from a syringe pump system (Harvard Apparatus Ltd., Edenbridge, U.K.) at 30 μL/min. The source temperature was kept at 90 °C. The MS was calibrated using either 12 pmol/μL horse heart myoglobin or a mixture of poly(ethylene glycol)s (PEG) with average MWs of 200, 400, 600, 900, and 1500. Peptides and globin samples were analyzed in the positive ionization mode as full scan or selected ion recording (SIR).

HPLC–MS. Samples were analyzed isocratically (solvent A, 82%; solvent B, 18%) on a Shandon Hypersil BDS C18 column (5 μm, 250 × 4.6 mm) using a Varian pump at 1 mL/min flow rate in combination with the Quattro-BQ MS. The tryptic peptide mixture derived from 2 to 3 mg/mL total protein was separated and the eluate passed through a splitter which directed approximately 100 μL/min flow directly to the electro-

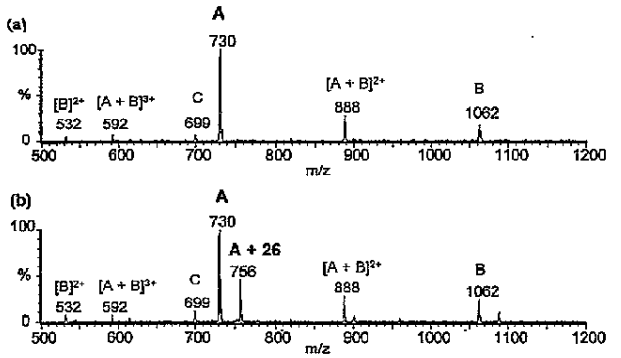


Figure 1. ESI-MS spectra of tryptic digests of unmodified 21-chain polypeptide (a) and modification by acetaldehyde (b) to give a mass increase of 26 Da on the N-terminal fragment of A.

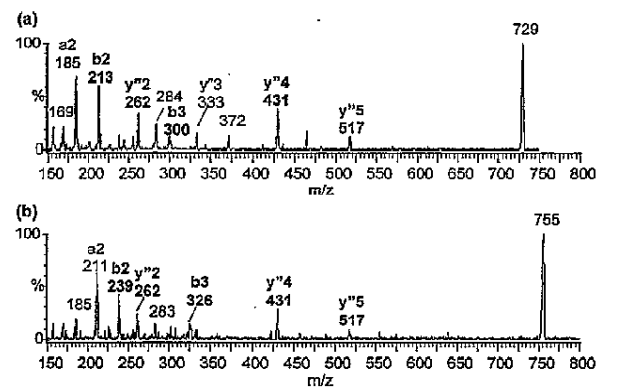


Figure 2. ESI-MS/MS spectra of the N-terminal heptapeptide fragment from the unmodified peptide (a) and modification by acetaldehyde (b), showing the ion series b and y″ which correspond to MS fragmentation at the peptide bond.

spray source and the remainder to waste. The electrospray source was operated at 145 °C. The mass spectrometer was set to monitor the four ions of interest (*m/z* 729.5, 755.5, 952.5, 978.5) by SIR.

Results

Site of Modification. The initial objective of the work was to characterize thoroughly the acetaldehyde adduct. This was achieved by the use of peptide A. Incubation of the peptides with excess acetaldehyde yielded stable adducts which were confirmed by MS as having a mass increment of 26 Da from the intact peptide. To find out which of the amino acids on the peptide had been modified, the peptide was digested with trypsin. Trypsin is a serine protease which cleaves at the C-terminal of arginine and lysine to give three fragments for this peptide. Electrospray mass spectrometry (ESI-MS) of the digest mixture showed that the predominant increase was on fraction A, the N-terminal heptapeptide (Figure 1). This fraction corresponds to the N-terminal α-chain of globin. The fractions were separated by reversed-phase HPLC and collected. Electrospray tandem MS (ESI-MS/MS) analysis of the heptapeptide (Figure 2) showed that adduction was predominantly at the N-terminal end of the peptide. N- and C-terminal fragments formed by cleavage of the amide bond between NH and CO are named b_n and y''_n ions according to the nomenclature of Roepstorff and Fohlman (*21*). The

Table 1. Fragment Ions from MS/MS Analysis[a]

N-terminus----CO- -$\overset{b}{\underset{y''}{\diagup\diagdown}}$- -NH----C-terminus

	b ion	b ion adduct	y ion	y ion adduct
Val	100.1	126.1		
Leu	**213.2**	**239.2**	630.3	630.3
Ser	**300.2**	**326.2**	**517.3**	**517.3**
Pro	397.2	423.3	**430.2**	**430.2**
Ala	468.3	494.3	**333.2**	333.2
Asp	583.3	609.3	**262.1**	**262.1**
Lys			147.1	147.1

[a] Bold figures correspond to observed ions for the unmodified (a) and modified (b) peptide from Figure 2.

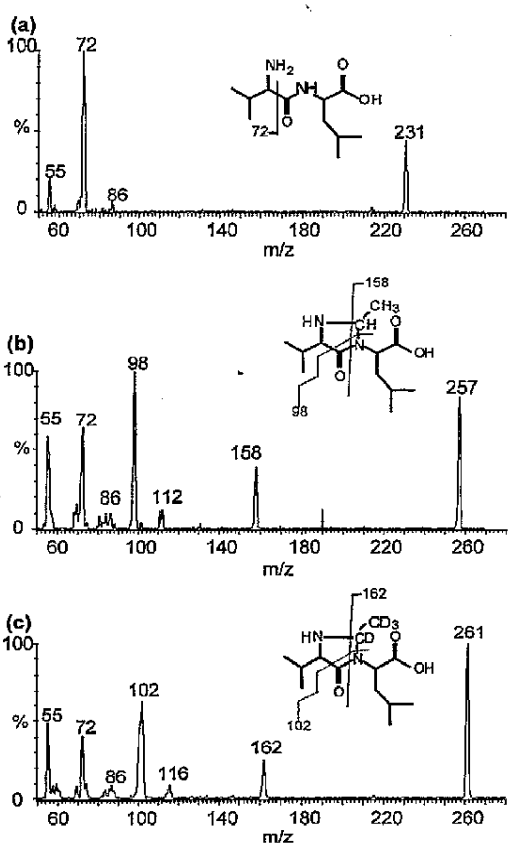


Figure 3. ESI-MS/MS spectra of the unmodified dipeptide (a), dipeptide modified with acetaldehyde-d_0 (CH_3CHO) (b), and dipeptide modified with acetaldehyde-d_4 (CD_3CDO) (c).

product ion spectrum of the $(M + H)^+$ peptide ion (*m/z* 729) contains ions of both the b and the y″ series. Table 1 shows the expected product ions from the heptapeptide and highlights the ions which are present in the spectrum.

MS/MS of Dipeptide Adducts. A commercially available dipeptide (peptide E) was used for a more detailed structural elucidation of the N-terminal adduct. Reaction of this peptide with acetaldehyde also yielded adducts which produced a mass increase of 26 Da in the MS. The MS/MS results of the dipeptide reactions with acetaldehyde and deuterium labeled acetaldehyde (CD_3CDO) support the proposed imidazolidinone structure. MS/MS fragmentation for the dipeptide and the acetaldehyde adducts are shown in Figure 3. Although these

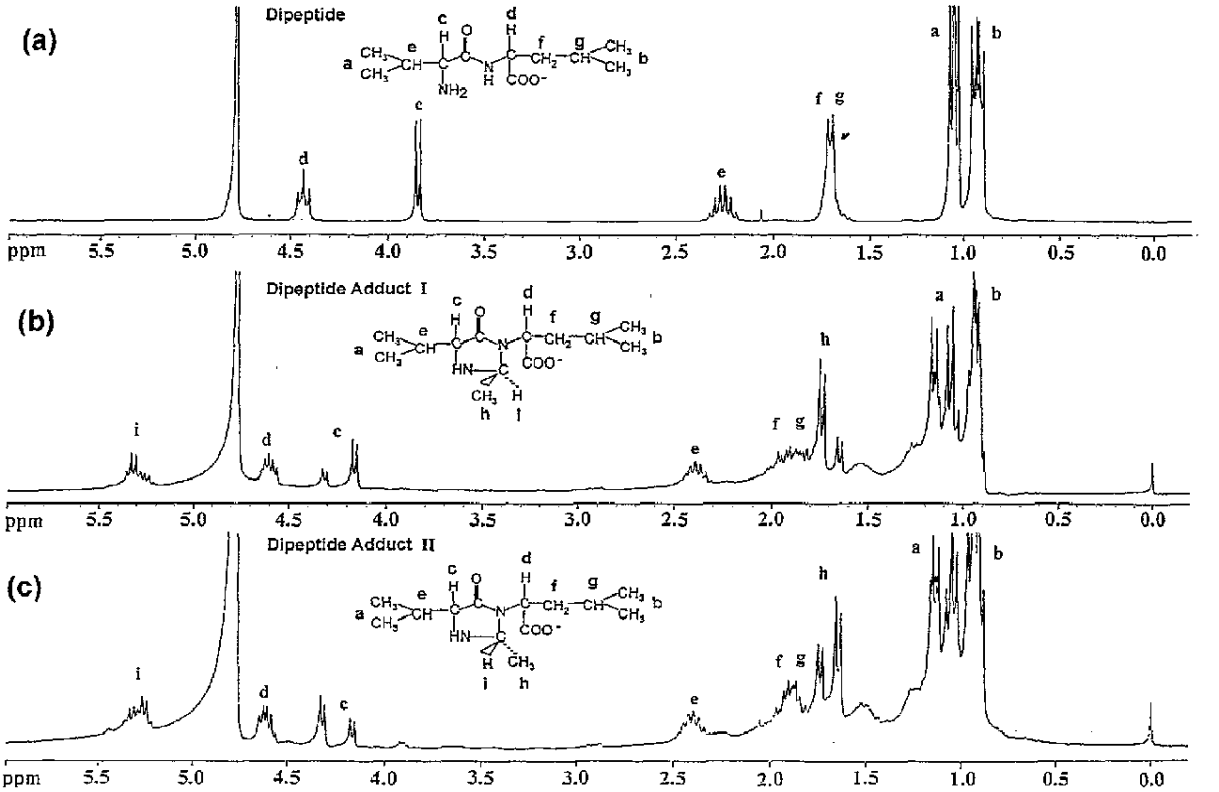


Figure 4. NMR spectra of unmodified dipeptide (a) and two of the partially resolved HPLC fractions from the dipeptide modification by acetaldehyde (CH_3CHO) (b and c).

patterns correspond to the expected ions from these structures, it is feasible, however, that similar fragments would also be expected from an unstable Schiff base adduct.

Structural Confirmation by NMR. Fractions from the HPLC separation of the products from the reaction of the dipeptide with acetaldehyde were manually collected over repeated injections. The dried fractions were redissolved in 0.1% TFA solution and reinjected. Fractions were collected and dried for further analysis. The resultant NMR spectrum in Figure 4 compares intact dipeptide and two other fractions which were not completely resolved on HPLC but show differences in their NMR. The main differences between the unmodified and the modified peptides are the presence of two new resonances: a quartet due to the CH group and a doublet due to the methyl group, both introduced by the imidazolidinone ring structure. Although the two HPLC fractions were not completely resolved from each other, the paired resonance peaks in the spectra indicate similar compounds with different chromatographic properties. Two-dimensional NMR performed on the mixture confirmed that they were diastereoisomeric products (data not shown).

Analysis of Globin Adducts by ESI-MS. Intact globin was analyzed using ESI-MS. The typical spectrum of globin consisted of a series of multiply charged ions related to ions bearing 12–18 charges. These multiply charged spectra can be converted using a deconvoluting algorithm (MaxEnt v2.1, Micromass, U.K.) to give true molecular masses. This was applied to samples of globin which had been incubated with varying concentrations of excess acetaldehyde at different times. Although it was difficult to differentiate between the two multiply charged ions in the initial spectrum due to poor resolution, the subsequent deconvolution revealed the differences between the globin chains. The converted spectra showed that some modification had occurred, especially to the β-chain after incubation with acetaldehyde (Figure 5). This was also confirmed in the reversed-phase HPLC of the intact globin samples. Separation of the intact globin provided two main peaks. Incubation with acetaldehyde altered the profile of these two peaks with modification of the β-chain more pronounced than the α-chain as also reported by Nguyen et al. (22).

Analysis of Tryptic Digests of Globin. The majority of the globin samples were monitored using ESI-MS after tryptic digestion before further purification; 29 peptides are expected from normal hemoglobin A (HbA) after tryptic digestion. Peptides derived from the enzymatic cleavage of the globin were also analyzed using the same techniques. The expected masses of the peptides from the tryptic digest of normal HfbA are shown in Table 2. Some of these peptides are shown in the spectra of Figure 6 (m/z range 650–1000). This compares the ESI mass spectrum of digest mixtures from control globin and from globin which has been incubated with excess acetaldehyde. The ESI of the digest mixtures shows matching protonated molecular ions corresponding to the N-terminals of the α-heptapeptide (m/z 729) and β-octapeptide (m/z 952) chains of HbA. Additional ions in the spectrum of the acetaldehyde incubated sample indicate the presence of adducted ions showing increments of 26 Da from the unmodified peptide molecular

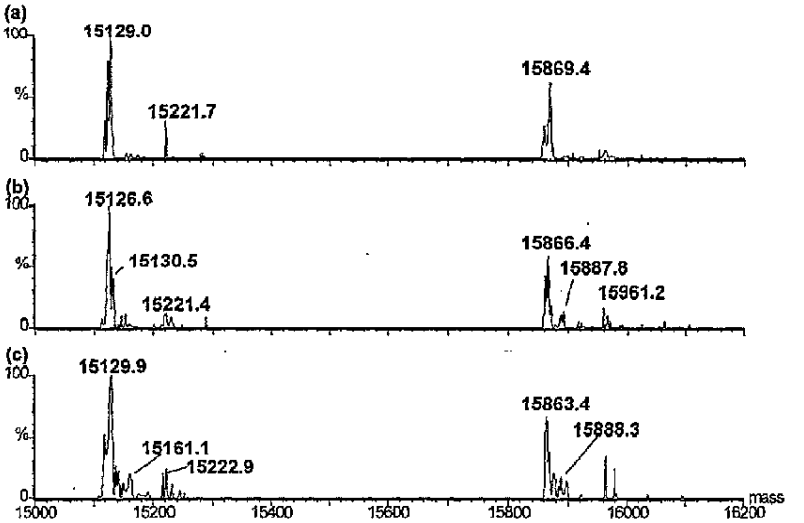


Figure 5. Series of deconvoluted spectra of globin samples incubated with 4× molar excess acetaldehyde for (a) 0, (b) 5.25, and (c) 24 h.

Table 2. List of Peptides Expected from Tryptic Digest of Normal Hemoglobin

fragment no.	theor (av)	(M + H)	(M + 2H)	(M + 3H)
	Digest Fragments of Globin α-Chain			
T1	728.84	729.85	365.43	243.96
T2	460.53	461.54	231.27	154.52
T3	531.61	532.62	266.81	178.21
T4	1529.63	1530.64	765.82	510.88
T5	1071.31	1072.31	536.66	358.11
T6	1834.02	1835.03	918.02	612.35
T7	397.43	398.44	199.73	133.49
T8	146.19	147.20	74.10	49.74
T9	2997.34	2998.35	1499.68	1000.12
T10	287.36	288.37	144.69	96.80
T11	817.94	818.95	409.98	273.65
T12	2968.51	2969.52	1485.27	990.51
T13	1252.47	1253.48	627.24	418.50
T14	337.38	338.39	169.70	113.47
	Digest Fragments of Globin β-Chain			
T1	952.08	953.08	477.05	318.37
T2	909.05	910.06	455.53	304.02
T3	1314.42	1315.43	658.22	439.15
T4	1274.53	1275.54	638.27	425.85
T5	2059.28	2060.29	1030.65	687.44
T6	245.32	246.33	123.67	82.78
T7	411.46	412.47	206.74	138.16
T8	146.19	147.20	74.10	49.74
T9	1669.90	1670.91	835.96	557.64
T10	1421.59	1422.60	711.81	474.87
T11	1126.24	1127.25	564.13	376.42
T12	1720.12	1721.12	861.07	574.38
T13	1306.48	1307.49	654.25	436.50
T14	1149.36	1150.37	575.69	384.13
T15	415.45	416.46	208.73	139.49

ions (*m/z* 755 and 978).

To confirm that these ions with increments of 26 Da were not due to other components in the crude digest mixture, HPLC purification was performed. Fractions were collected manually from repeated injections. The fractions were numbered according to their order of elution. The dried samples were then reanalyzed on ESI-MS. The heptapeptide ion of *m/z* 729 and the octapeptide at *m/z* 952 were identified in two of the fractions. The main ions in a third fraction from the exposed sample but not in the control contained ions (*m/z* 755 and 978) which correspond to the modified heptapeptide and octapeptide.

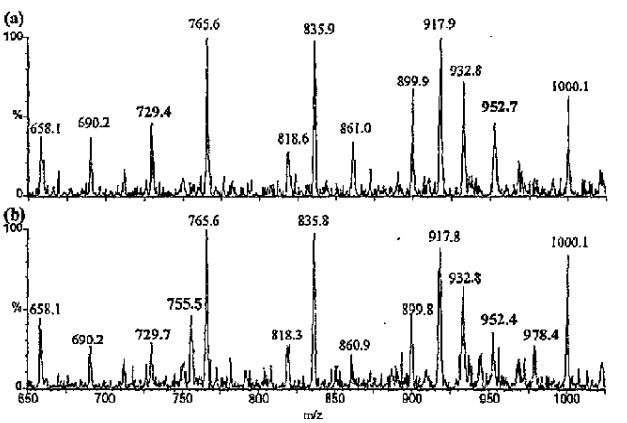


Figure 6. ESI-MS showing tryptic digest of control (a) and exposed globin (b).

MS/MS of Tryptic Digests of Globin. MS/MS was used to confirm whether these adducts were due to the imidazolidinone structure and not to some interfering ions from the digest mixture. The product ion spectrum of the $(M + H)^+$ of peptide B (*m/z* 729) contains ions of both the b and the y″ series. This is similar to the product ion spectrum produced by the heptapeptide from the tryptic digest of peptide A (Figure 2). A similar but noisier spectrum was obtained for both the unmodified peptide (*m/z* 729) and the modified peptide (*m/z* 755) ions in the tryptic digest mixture of globin. The most intense ion at *m/z* 430, which corresponds to the fragment also found in the heptapeptide standard, was detected and hence confirmed the identity of the observed $(M + H + 26)^+$ ion. The tryptic digest product of peptide D, which corresponds to the octapeptide, elutes at the same retention time as the digest fragment from the globin.

HPLC–MS of Globin Digests. SIR was used to measure the N-terminal peptides and their adducts. The ions 729.5, 755.5, 952.5, and 978.5 were monitored on the MS to provide a sensitive analysis of these peptides from the crude tryptic digest mixtures. Although specificity is achieved by the use of SIR, the presence of interfering ions of the same *m/z* was still possible. An on-line chromatographic separation preceding MS analy-

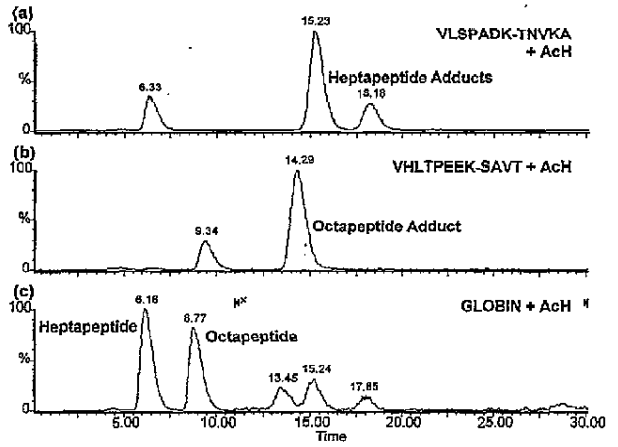


Figure 7. On-line (LC–MS) TIC of the tryptic digests of (a) peptide C, (b) peptide D, and (c) globin after incubation with excess acetaldehyde.

sis of the tryptic digest mixtures of globin, although adding an extra step, would aid in eliminating this problem as well as in assisting in improved specificity of the assay. In Figure 7, a comparison of the total ion chromatograms (TIC) of the tryptic digests of the 12-mer peptides (C and D) and globin after incubation with excess acetaldehyde demonstrates the application of this method. The peaks were identified with the aid of the pure heptapeptide standard and the digested 12-mer control peptides. Both the pure heptapeptide and the digested α-chain 12-mer gave peaks at the 6.2-min region. The peaks appearing at approximately 9 min are due to the octapeptide. The octapeptide adduct appears at approximately 14 min. Both the globin and α-chain peptide gave two new peaks which are probably due to the diastereoisomeric adduct products. Because of concern at the possible loss of the adducts during the globin workup, the assay was also carried out following the incubation of freshly collected heparinized blood with acetaldehyde at 0, 0.2, 2, and 20 mM. After the usual workup and analysis by on-line ESI-MS, only the samples from the 2 and 20 mM incubations gave peaks that were due to the expected adducts.

Discussion

Acetaldehyde forms both stable and unstable adducts with various proteins as mentioned earlier. In this report, we have demonstrated the presence of adducts formed by incubation of acetaldehyde with various peptides and human globin at the N-terminal amino group. The initial identification of the position of adduction was made using a synthetic 21-mer peptide which contains one of each of the common amino acids. Reaction of this peptide with acetaldehyde followed by digestion with trypsin and analysis of the fragments by tandem MS resulted in the identification of the N-terminal valine as the position of major adduction. A dipeptide was used to characterize the actual structure at the N-terminus. Analysis of samples incubated with acetaldehyde showed that diastereoisomers of imidazolidinone adducts had been formed. This structure was originally suggested by San George and Hoberman (*15*) to arise as a result of spontaneous rearrangement of the unstable Schiff base. We have also developed an HPLC–MS method to measure simultaneously the stable imidazolidinone adducts of the α- and β-terminals of human globin. Reaction of increasing concentrations of acetaldehyde afforded a linear dose–response relationship with the formation of these adducts in globin. There were however differences in the reactivities of the α- and β-termini as also reported by Nguyen et al. (*22*) with most of the modifications taking place on the β-chain. Both chains terminate in valines, and the preference for the β-chain can be explained by the possible steric effects of neighboring amino acids on the conformation at the termini.

As described in the Introduction, early damage due to alcohol misuse is not easy to determine before manifestation of ill health, and although the potential offered by protein adducts as biomarkers has been widely studied for many exposures, little progress has yet been made with respect to alcohol. Many approaches to developing this biomarker have been attempted. For example, the use of hemoglobin acetaldehyde adducts for monitoring alcohol exposure has been suggested to be analogous to that of glycated hemoglobin (HbA_{1c}) for determining the degree of glycemia in diabetics. Sillanaukee et al. (*23*) proposed the use of the ratio of the fast eluting HbA_{1ach}/HbA_{1c} as a marker, using cation-exchange chromatography. Since then a number of improvements to this separation have been made. However, the technique fails to differentiate significantly between controls and alcoholics. Although observed increases in the minor hemoglobin component are present, there have not been measured differences with normal subjects (*23*). Lucas et al. (*24*) found little difference between samples from alcoholic and controls when they measured the acetaldehyde released after acid hydrolysis of their samples. This chromatographic technique has an advantage of being indicative of the overall status of the minor hemoglobin. However there are no means of differentiating alterations caused by other chemicals in the body. Hence even if the chromatography was sufficiently improved to differentiate the available sample pools, the lack of specificity would still apply. Any alternative technique would however need to be both specific and sensitive, as the level of acetaldehyde adducts is so low (<0.2%) in comparison to unmodified protein (*25*). The level of acetaldehyde circulating in the blood is highly variable and has been measured to be in the range from 0 to 200 μM (teetotalers to alcoholics).

A very sensitive technique is therefore required for the detection of the resulting adducts. Immunological techniques have been explored as potential methods, and Lin et al. (*25*), for example, used a sandwich ELISA technique to differentiate between control and alcoholic samples. The major site of adduction of acetaldehyde on the hemoglobin is believed to be the center of the β-chain where several lysine residues are clustered with the ε-amino group being the site of this modification (*14, 19, 25*). Adduction with lysyl groups produces unstable products (Schiff bases) and hence would not be a suitable marker. Stabilization of these adducts can be achieved by a reduction step using borohydride in vitro. Braun et al. (*26*) have used ^{13}C NMR and Raman spectroscopy to confirm the formation of stable Schiff base adducts after 1-h incubations with the N-terminal octapeptide from the β-chain of sickle globin.

The method described in the present report has the advantage in that a further reaction is not required to give the stable adducts. Using the on-line HPLC–MS

technique described above, it has been possible to detect adducts formed after incubation of whole blood with 2 mM acetaldehyde. This represents our limit of detection at present, and in preliminary studies, we have not been able to detect these adducts in samples from two chronic alcoholics that we have analyzed. Several possibilities exist for improving the sensitivity of the method, and they are being actively pursued. For example, the use of capillary columns linked to MS instruments together with nanospray introduction of samples to the MS has the potential of increasing the relative sensitivities of assays by a factor of several thousand in comparison to conventional HPLC columns (27). The unambiguous detection and quantitation of stable acetaldehyde adducts with human globin provides a firm base for the development of a biomarker of alcohol intake in humans.

Acknowledgment. We thank Dr. Gavain Sweetman and Mr. John Lamb for their advice and help on electrospray mass spectrometry. We also thank Dr. Ron Lawrence and Miss Rebekah Jukes for assistance with the NMR and Mrs. Beryl Tracey for assistance with the HPLC. We thank Prof. T. J. Peters (Kings College School of Medicine and Dentistry, London) for the supply of human samples. This work was supported in part by the Ministry of Agriculture, Fisheries and Food (MAFF), Contract 1A025.

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TX970169Z